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Polysaccharide microarray technology for the detection of *Burkholderia pseudomallei* and *Burkholderia mallei* antibodies

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Abstract

A polysaccharide microarray platform was prepared by immobilizing *Burkholderia pseudomallei* and *Burkholderia mallei* polysaccharides. This polysaccharide array was tested with success for detecting *B. pseudomallei* and *B. mallei* serum (human and animal) antibodies. The advantages of this microarray technology over the current serodiagnosis of the above bacterial infections were discussed.

Keywords: Polysaccharide microarrays; *Burkholderia pseudomallei*; *Burkholderia mallei*; Glanders; Melioidosis

1. Introduction

There has been a great deal of emphasis on the development of DNA and protein microarrays recently (Dietrich, 2003; Schweiter et al., 2003). However, DNA and protein microarrays fail to address glycosylation, which is a posttranslational modification. Many Gram-negative bacteria contain structurally unique polysaccharides, namely, capsular polysaccharide and lipopolysaccharide (LPS) *O*-antigen, that are often pathogen specific (Raetz and Whitfield, 2002). In other words, these polysaccharides are "signature polysaccharides" of their respective bacteria. It is possible to immobilize microbial polysaccharides on glass slides for use as microarrays. Sera from infected or immunized animals or humans could be used to bind to the arrays. Polysaccharide microarray technology can be used as a novel approach for the diagnosis of bacterial infections (Feizi et al., 2003; Hirabayashi, 2002; Feizi and Mulloy, 2003; Love and Seeberger, 2004; Thirumalpura et al., 2005; Blixt et al., 2004; Willats et al., 2002). Indeed, the employment of carbohydrate microarrays using immobilized microbial polysaccharides (from *Klebsiella*—type 7, K11, K13, K21), *Pneumococcus* (type C-type VII, type XIV), meningococcus group B, *Haemophilus influenzae* type A, and *Escherichia coli* K92 has been demonstrated recently to diagnose microbial infections (Wang et al., 2002). The application of glycan arrays for the identification of immunologic targets for the viral pathogen severe acute respiratory syndrome coronavirus was also reported (Wang and Lu, 2004). Therefore, carbohydrate and polysaccharide microarrays are emerging technologies that are promising alternatives to DNA and protein microarrays.

In the present study, we generated polysaccharide microarrays by surface immobilization onto glass slides using the polysaccharides derived from *Burkholderia pseudomallei* and *Burkholderia mallei* and then probing with antisera containing antibodies specific for a capsular polysaccharide common to both pathogens. *B. pseudomallei* and *B. mallei* infections are known as melioidosis and glanders, respectively (Cheng and Currie, 2005; Waag and DeShazer, 2004; Woods et al., 1999). Melioidosis is endemic primarily in Southeast Asia and Northern Australia. Glanders is naturally found in equines, which occasionally transmit the infection to humans. Both *B. mallei* and *B. pseudomallei* are Centers for Disease Control and Prevention (CDC) category B biothreat agents (Rotz et al., 2002). The polysaccharide microarray described in this study was successfully used to detect anticapsule antibodies in the serum of a rabbit immunized with purified *B. pseudomallei* capsular polysaccharide and in the convalescent serum from a human infected with *B. mallei* (Srinivasan et al., 2001). The application of this array

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14. ABSTRACT

A polysaccharide microarray platform was prepared by immobilizing Burkholderia pseudomallei and Burkholderia mallei polysaccharides. This polysaccharide array was tested with success for detecting B. pseudomallei and B. mallei serum (human and animal) antibodies. The advantages of this microarray technology over the current serodiagnosis of the above bacterial infections were discussed.

15. SUBJECT TERMS

Burkholderia pseudomallei, mallei, polysaccharide microarrays, gram-negative bacterial pathogens, glanders, melioidosis

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technology could be extended to the diagnosis of other Gram-negative bacterial infections.

1.1. Bacterial strains and growth conditions

The bacterial strains used in this study were *B. pseudomallei* 1026b (DeShazer et al., 1997), *B. pseudomallei* 576 (Atkins et al., 2002a), *B. pseudomallei* SRM117 (DeShazer et al., 1998), *B. mallei* ATCC 23344. All strains produce a common capsular polysaccharide, but *B. pseudomallei* 576 produces an atypical LPS *O*-antigen (Atkins et al., 2002b), and *B. pseudomallei* SRM117 is an LPS *O*-antigen mutant. *B. pseudomallei* strains were grown at 37 °C on Luria–Bertani (LB) agar (Lennox, St. Louis, MO) or in LB broth (Lennox), and *B. mallei* strain was grown at 37 °C on LB agar or in LB broth containing 4% glycerol. Under these culture conditions, both *B. pseudomallei* and *B. mallei* had been shown (Burtnick et al., 2002) to produce capsular polysaccharide and LPS.

1.2. Polysaccharide isolation

Polysaccharides (capsular polysaccharide and LPS) were extracted from the bacterial cell pellet by hot phenol extraction method and purified after digestion with DNase, RNase, and proteinase K digestion essentially by the methods described previously (Burtnick et al., 2002, Brett and Woods, 1996). The isolated polysaccharides were hydrolyzed (2% acetic acid, 100 °C, 2 h) to release the lipid A moiety, which was removed by centrifugal filtration (Amicon centrifugal device MW cutoff 5K).

1.3. Polyclonal antibody and human serum

The polyclonal rabbit antibody raised against *B. pseudomallei* capsular polysaccharide–flagellin protein conjugate was kindly provided by D.E. Woods, Calgary, Alberta, Canada. The details of the preparation and specificity of this antibody were previously published (Reckseidler et al., 2001). Human convalescent serum was collected from an individual after an accidental laboratory exposure to *B. mallei* and presented with clinical symptoms of glanders infection (Srinivasan et al., 2001). Normal preinfection serum was also collected. The time of collection of serum after exposure is approximately 2 months. A minimal risk protocol to collect the blood sample was approved by the institutional review board at the United States Army Medical Research Institute of Infectious Diseases (USAMRIID) (Human Use Committee HP 06-02). The donor was provided with informed consent and met the eligibility criteria.

1.4. Polysaccharide microarray

The polysaccharides were converted to glycosylamines in the presence of ammonium acetate by reductive amination (Spiro and Spiro, 1992). Briefly, to the dry polysaccharides in Reacti-Vials (Pierce, Rockford, IL), 50 μL of 0.3 mol/L sodium cyanoborohydride in 2 mol/L ammonium acetate, pH 6.0, was added, and capped vials were incubated at 105 °C for 4 h. Distilled deionized water (100 μL) and 40 μL of 6 mol/L formic acid were added, and the contents were dried in the Speed Vac at 45 °C. Ammonium acetate in the reaction mixture was removed by addition of methanol (0.5 mL) 3 times followed by repeated drying in the Speed Vac at 45 °C. The glycosylamine derivatives of the polysaccharides were reconstituted in 1.0 mL distilled deionized water. The neutral sugar contents of the polysaccharides were estimated by phenol–sulfuric acid method in microplate format (Masuko et al., 2005) using L-rhamnose as the standard. Glycosylamine polysaccharides were printed on glass slides (Super Epoxy in 16-wells, NUNC, Rochester, NY) using a robotic microarrayer (VIRTEK, Chip Writer Pro, Bio-Rad, Hercules, CA). Custom printing was carried out by Kam Tek, Gaithersburg, MD. The polysaccharides were reconstituted in Tris-buffered saline (TBS) (25 mmol/L Tris, 0.15 mol/L NaCl, pH 7.2) and arrayed at 6 different dilutions in triplicates. The original concentrations of polysaccharides from ATCC 23344, SRM117, 1026b, and 576 (micromoles of rhamnose equivalents per milliliter) were 3.6, 16.0, 3.6, and 3.5, respectively. We also used inulin (Sigma) as a negative control (2.8 μmol glucose equivalents per milliliter). The slides were blocked with 200 μL of 2% (wt/vol) bovine serum albumin (Sigma, St. Louis, MO) in TBS for 1 h at room temperature. After 1 h of incubation at room temperature, with 100 μL of a 1:1000 dilution of rabbit polyclonal serum (specific for *B. pseudomallei* capsular polysaccharide) in TBS, the slides were washed 3 times with TBS containing 0.1% Tween 20 at 5 min intervals. Serum from a case of human glanders was also used, but at a dilution of 1:5000 in TBS. Cy3- and Cy5-labeled antirabbit IgG (H + L) secondary antibodies were purchased from Amersham Biosciences (Piscataway, NJ). Antihuman IgG (H + L) (KPL, Gaithersburg, MD) secondary antibody was labeled with Cy3 and Cy5 bifunctional NHS (*N*-hydroxysuccinimide) ester dyes using Amersham Biosciences kit. The secondary antibodies were used at a 1:1000 dilution in TBS. After washing, the slides were scanned for fluorescence at 635 nm (Cy3) and at

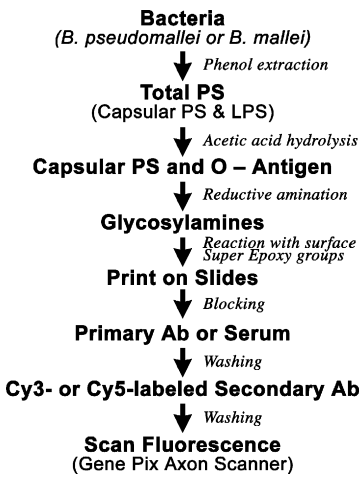

Fig. 1. Flow chart of the generation of *Burkholderia* polysaccharide microarray.

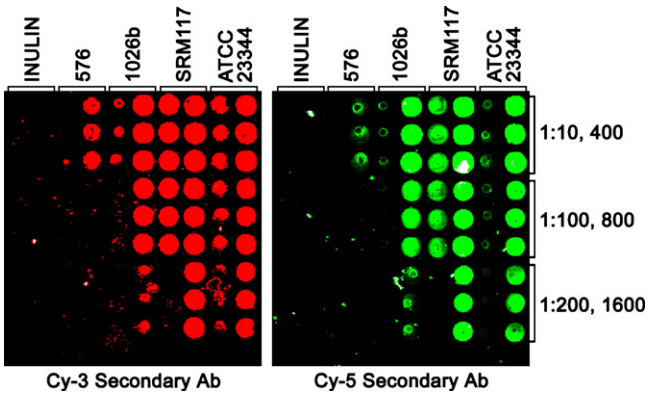

Fig. 2. Immunoreactivity of *Burkholderia* polysaccharide microarray with polyclonal rabbit antiserum raised against purified *B. pseudomallei* capsular polysaccharide. The concentrations of polysaccharides were in decreasing order in triplicates: 1:10, 1:100, and 1:200 (right lanes); 1:400, 1:800, and 1:1600 (left lanes). Cy3- and Cy5-labeled secondary antibodies were reacted with the microarrays as described in the text. No positive spots were seen (diagram not shown) on probing with normal serum.

532 nm (Cy5) with a Gene Pix 4000B Axon Scanner, using the software Gene Pix Pro 5.1.

The general scheme of approach of polysaccharide microarray is outlined in Fig. 1. A mixture of capsular polysaccharides and *O*-antigen saccharides was isolated from different bacterial strains including ATCC 23344, 1026b, and 576. SRM117 is an LPS mutant that produces capsular polysaccharide but not *O*-antigen saccharides. The presence of capsular polysaccharide in these polysaccharide preparations was confirmed by Western blotting (diagram not shown) using a polyclonal antibody specific for the capsular polysaccharide (Burtnick et al. 2002). The polysaccharides were converted to glycosylamines by reductive amination with sodium cyanoborohydride in the presence of ammonium acetate. To investigate whether immobilized polysaccharide macromolecules preserve their antigenic determinants, we printed polysaccharide (capsular polysaccharide and LPS *O*-antigens) preparations from different strains of *B. mallei* and *B. pseudomallei* on glass slides. The slides were probed with a polyclonal antibody specific to capsular polysaccharide. Both *B. mallei* and *B. pseudomallei* are encapsulated with a polysaccharide of the same structure. The capsular polysaccharide is a homopolymer of 1,3-linked 2-*O*-acetyl-6-deoxy-β-D *manno*-heptopyranose-(1 (Perry et al., 1995; Knirel et al., 1992). All the strains used in the study had been shown to contain this capsular polysaccharide (Atkins et al., 2002a; DeShazer et al., 2001; Reckseidler et al., 2001; DeShazer et al., 1998). It is apparent that the immunoreactivity of this polysaccharide is preserved even after surface immobilization, as evidenced by their recognition and reactivity to the antibody specific for the capsular polysaccharide (Fig. 2). It is not clear why this antibody gave weaker signal against the polysaccharide derived from 576 as compared with the other strains. As expected, the rabbit antiserum did not react with inulin, the polysaccharide used as a negative control.

Furthermore, using this microarray, we are able to detect capsular polysaccharide antibodies in the serum of a human patient (Srinivasan et al., 2001) exposed to *B. mallei* infection. Although these antibodies were present in the convalescent serum, they were absent in preinfection serum from the same patient (Fig. 3). Possibly, the glanders patient serum also contains antibodies against LPS *O*-antigens, but this requires further investigation.

In the present study, we are able to demonstrate the usefulness of polysaccharide microarray technology for the detection of serum antibodies against *B. mallei* and *B. pseudomallei*. An indirect hemagglutination and a complement fixation (CF) test had been the routine procedure for the serologic diagnosis of human melioidosis caused by *B. pseudomallei* infection (Alexander et al., 1970; Wuthiekanum et al., 2006). *B. mallei* infections (glanders) in horses had been serologically diagnosed employing CF test (Marek and Manninger, 1945). Both indirect hemagglutination and CF tests are based on crude whole-cell preparation or extracts of the bacteria, and therefore, the potential for false-positive serodiagnosis cannot be ruled out (Marek and Manninger, 1945; Wernery et al., 2004, 2005). Neubauer

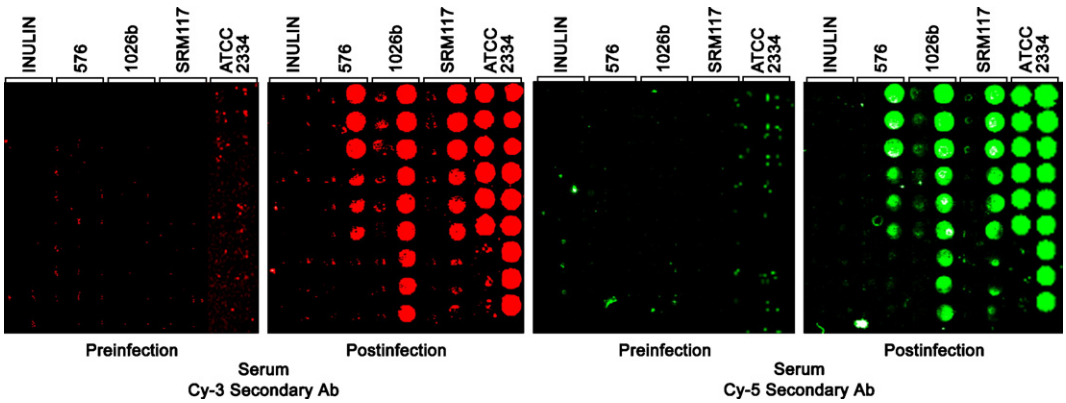

Fig. 3. Typical Immunoreactivity of *Burkholderia* polysaccharide microarray with the serum of a human glanders patient. The microarrays were reacted with preinfection and postinfection sera from a human glanders patient at 1:5000 dilution. Cy3- and Cy5-labeled secondary antibody was reacted with the microarrays as described in the text. Immunoreactivity was also seen at 1:10.000 and 1:40 000 dilutions as well (diagram not shown).

et al. (2005) suggested the importance of serologic tests using well-characterized antigens in the place of crude bacterial preparations to avoid the diagnosis of false positives. In the present study, we used well-characterized antigens (capsular polysaccharide and *O*-antigen saccharides) in the microarray, and this should circumvent the problems of false positives in future serodiagnosis of *B. mallei* and *B. pseudomallei* infections.

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